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Ca: Mg Ratio, Medium-chain Fatty Acids, and the Gut Microbiome

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The Personalized Prevention of Colorectal Cancer Trial (PPCCT) was registered at clinicaltrials.gov as NCT01105169.

Abstract

Background & aims—Ketogenic medium-chain-fatty acids (MCFAs) with profound health benefits are commonly found in dairy products, palm kernel oil and coconut oil. We hypothesize that magnesium (Mg) supplementation leads to enhanced gut microbial production of MCFAs and, in turn, increased circulating MCFAs levels.

Methods—We tested this hypothesis in the Personalized Prevention of Colorectal Cancer Trial (PPCCT) (NCT01105169), a double-blind 2 × 2 factorial randomized controlled trial enrolling 240 participants. Six 24-hour dietary recalls were performed for all participants at the baseline and during the intervention period. Based on the baseline 24-hour dietary recalls, the Mg treatment used a personalized dose of Mg supplementation that would reduce the calcium (Ca): Mg intake ratio to around 2.3. We measured plasma MCFAs, sugars, ketone bodies and tricarboxylic acid cycle (TCA cycle) metabolites using the Metabolon's global Precision Metabolomics™ LC-MS platform. Whole-genome shotgun metagenomics (WGS) sequencing was performed to assess microbiota in stool samples, rectal swabs, and rectal biopsies.

Results—Personalized Mg treatment (mean dose 216.5 mg/day with a range from 77.25 mg/day to 389.55 mg/day) significantly increased the plasma levels of C7:0, C8:0, and combined C7:0 and C8:0 by 18.45%, 25.28%, and 24.20%, respectively, compared to 14.15%, 10.12%, and 12.62% decreases in the placebo arm. The effects remain significant after adjusting for age, sex, race and baseline level (P=0.0126, P=0.0162, and P=0.0031, respectively) and FDR correction at 0.05 (q=0.0324 for both C7:0 and C8:0). Mg treatment significantly reduced the plasma level of sucrose compared to the placebo arm (P=0.0036 for multivariable-adjusted and P=0.0216 for additional FDR correction model) whereas alterations in daily intakes of sucrose, fructose, glucose, maltose and C8:0 from baseline to the end of trial did not differ between two arms. Mediation analysis showed that combined C7:0 and C8:0 partially mediated the effects of Mg treatment on total and individual ketone bodies (P for indirect effect =0.0045, 0.0043, 0.03, respectively). The changes in plasma levels of C7:0 and C8:0 were significantly and positively correlated with the alterations in stool microbiome α diversity (r=0.51, p=0.0023 and r=0.34, p=0.0497, respectively) as well as in stool abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene producing C7:0 (r=0.46, p=0.0067) and C8:0 (r=0.49, p=0.003), respectively, following Mg treatment.

Conclusions—Optimizing Ca:Mg intake ratios to around 2.3 through 12-week personalized Mg supplementation leads to increased circulating levels of MCFAs (i.e. C7:0 and C8:0), which is attributed to enhanced production from gut microbial fermentation and, maybe, sucrose consumption.

Keywords

Ca:Mg intake ratio; medium-chain fatty acids; sucrose consumption; microbial alpha diversity; ketone body; randomized controlled trial

Introduction

Unlike long-chain fatty acids (LCFAs), medium-chain fatty acids (MCFAs), a class of saturated fatty acids with 6 to 12 carbon atoms, are absorbed directly by intestinal

enterocytes and rapidly transferred by the portal vein to the liver [1]. Further, MCFAs cross the blood-brain barrier and permeate the mitochondrial membrane independently of the carnitine transport system and undergo preferential β-oxidation, suggesting a high efficiency in absorption, transportation and utilization for energy production [1]. In the agriculture industry, MCFAs have emerged as a promising intervention to improve survival of baby animals due to their inhibitory effects against viral and bacterial pathogens and immunomodulatory activities [2]. Furthermore, evidence from human studies suggests that MCFAs have potentials to treat a wide range of neurological and metabolic disorders such as drug-resistant epilepsy [3,4], obesity, type 2 diabetes [5,6], nonalcoholic fatty liver disease (NAFLD) [7], cancer [8,9] and Alzheimer's disease [10,11].

The natural sources of even-chain MCFAs are dairy products and tropical oils such as palm kernel and coconut oil [12]. Additionally, during industrial ethanolic fermentation, yeasts produce MCFA ethyl esters such as ethyl caproate (C6:0) and ethyl caprylate (C8:0) as important flavor-active compounds[13,14]. Furthermore, a variety of bacteria, mostly anaerobic bacteria, and plants are equipped with acyl-acyl carrier protein (ACP) thioesterases, which are essential chain-terminating enzymes during *de novo* synthesis of fatty acids, including MCFAs [15]. Anaerobic bacteria including *Megasphaera elsdenii* and *Clostridium sp. BS-1* have been shown to produce MCFAs from sucrose or D-galactitol [16,17]. In *vitro* studies further indicate that metabolic engineered *Escherichia coli* resulted in substantially increased production of not only even-, but also odd-chain MCFAs like heptanoate (C7:0) [18], through endogenous expression of acyl-ACP thioesterases from *Acinetobacter baylyi* [19] and *Umbellularia californica* (BTE) [19,20]. However, no human or animal studies have been conducted to investigate whether gut microbiota can generate MCFAs.

Ionized calcium (Ca^{2+}) and magnesium (Mg^{2+}) play an important role in determining fermentation performance in yeasts [21,22]. Previous studies consistently found that optimizing the Ca:Mg ratio by supplementing Mg^{2+} in growth media enhanced the rate of sugar consumption and production yield in industrial yeast fermentation processes [22–24]. Furthermore, the thioesterase enzymes for MCFAs production are highly conserved across different species [25] and their activity is Mg-dependent [26]. Nevertheless, no studies have examined whether optimizing the Ca:Mg ratios by Mg supplementation affects microbial production of MCFAs in *in vivo* or human studies. In this regard, we hypothesized that optimized dietary Ca:Mg intake ratios could lead to increased circulating level of MCFAs (i.e. C6:0, C7:0 and C8:0) which can be attributed to enhanced production from gut microbial fermentation and sucrose consumption. We tested this hypothesis in the first precision-based randomized trial "Personalized Prevention of Colorectal Cancer Trial" (PPCCT) in which Ca:Mg ratios in diets were optimized to around 2.3 using personized supplementation of Mg [27,28].

Methods

Study Participants

The PPCCT (registered at clinicaltrials.gov as NCT01105169) is a double-blind 2×2 factorial randomized controlled trial conducted at Vanderbilt University Medical Center,

Nashville, TN. The detailed study design has been reported previously [27]. In brief, eligible participants were those aged 40 to 85 years old who had a calcium (Ca) intake ≥ 700 mg/day and < 2000 mg/day and in whom the Ca:Mg intake ratio was ≥ 2.6.

Dietary intake assessment

Dietary intake data were collected and analyzed using Nutrition Data System for Research (NDSR) Software Food and Nutrient Database developed at University of Minnesota Nutrition Coordinating Center (NCC) that facilitates the collection of recalls in a standardized fashion (http://www.ncc.umn.edu/products/). The NDSR program automatically generates intake of nutrients per day. It also includes a dietary supplement assessment module so that nutrient intake from both food and supplemental sources can be captured and quantified. Finally, the NDSR software is commonly used in clinical settings and in nutrition research, and NDSR software has been demonstrated to have high inter-rater reliability and good construct validity for identifying individual dietary intake patterns [29].

Interventions and precision-based dosing strategy

Eligible participants were assigned to Mg treatment or placebo according to the randomization schedule. Two 24-h dietary recalls were performed for all participants at the baseline of the PPCCT to determine Ca and Mg intakes. Based on their baseline intakes of Ca and Mg as well as their Ca: Mg intake ratio, each participant was assigned to a customized dose of Mg supplementation that would reduce the Ca: Mg intake ratio to ~2.3, as suggested by several previous studies [30–33]. The mean recorded intake from two 24-h recalls was used to estimate the baseline intakes of Ca and Mg, and the Ca: Mg ratio. Mg glycinate and identical-appearing placebo (i.e. microcrystalline cellulose) were provided in capsules. Four additional 24-hour dietary recalls were conducted for all participants during the intervention period with two during weeks 1 to 6 and the other two during weeks 7 to 12.

Among 250 randomized participants who started treatments, 239 completed the trial and one had donated blood at baseline and at the end of the trial. Therefore, 240 participants had blood collected at baseline and at the end of the trial. The mean daily dose of personalized Mg supplementation was 205.52 mg, with a range from 77.25 to 389.55 mg. Compliance with the pill regimen were very high for both the treatment and placebo arms based on pill counts (96.70% ± 7.61% and 96.60% ± 6.87%, respectively; $P_{\text{difference}}$= 0.91). The mean ± SD Ca:Mg ratios for the treatment and placebo arms after administering Mg and placebo supplementation were 2.27 ± 0.13 and 3.87 ± 1.46, respectively ($P_{\text{difference}}$<0.0001), at baseline and remained stable at 2.13 ± 0.68 and 3.51 ± 1.32, respectively ($P_{\text{difference}}$<0.0001), based on the four 24-hour dietary recalls conducted over the 12-week period of the trial. In this ancillary study, 68 participants were available for plasma metabolites measurement who were balanced on treatment (34 in the treatment and 34 in the placebo arm) and sex.

Measurement of Plasma Metabolites

For all participants, fast blood samples collected pre-intervention and at the end of the trial (12 weeks) will be assayed. Fasting blood samples were stored on ice immediately until processed within 6 hours. All samples were placed in long-term storage in −80°C

freezers [27]. Plasma samples were organized in treatment-placebo sets (4 samples in each set: 2 from pre- and 2 from post-treatment). Samples were shipped overnight on dry ice to Metabolon Inc. To control for batch variability, samples from each set were analyzed in the same run. Lab staff were blinded to sample status. We measured plasma MCFAs, sugars, ketone bodies and tricarboxylic acid cycle (TCA cycle) metabolites using the Metabolon's global Precision Metabolomics™, ultrahigh performance liquid chromatography (UHPLC)-mass spectrometry (MS) as reported in detail previously [28]. Lab staff were blinded to sample status. Sucrose consists of one fructose and one glucose and undergoes hydrolyzation by the enzyme sucrase present in the small intestine or spill over to the liver and to the colonic microbiota [34,35]. Recent controlled feeding studies found that the 24-hour urinary excretion of combined free fructose and the fructose from sucrose is significantly correlated with total sugar intakes, highlighting the importance of combined free fructose and the fructose from sucrose as objective biomarkers of total sugar intakes [34,36,37]. Thus, we calculated total fructose load using the sum of free fructose and the fructose from sucrose in circulation levels, and total glucose load using the sum of free glucose and the glucose from sucrose.

Microbiome Genome Sequencing and Shannon Diversity

The stool, rectal swab and mucosal tissue samples were collected at home or in-person visits at baseline and at the end of the trial. All the samples were frozen at −80 °C until use. For DNA isolation, samples were transferred to a 2 ml tube containing 200 mg of ≤106 μm glass beads (Sigma, St. Louis, MO, USA) and 0.3 ml of Qiagen ATL buffer (Qiagen, Valencia, CA, USA), supplemented with lysozyme (20 mg/ml) (Thermo Fisher Scientific, Grand Island, NY, USA). The suspension was incubated at 37°C for 1 h with occasional agitation and then supplemented with 600IU of proteinase K and incubated at 60°C for 1 h. Finally, 0.3 ml of Qiagen AL (Qiagen, Valencia, CA, USA) buffer were added and incubated at 70°C for 10 minutes, followed with 3 min bead beating in a Qiagen TissueLyser II (Qiagen, Valencia, CA, USA) at 30Hz. After a brief centrifugation, supernatants were transferred to a new tube containing 0.3 ml of ethanol. DNA was purified using a standard on-column purification method with Qiagen buffers AW1 and AW2 (Qiagen, Valencia, CA, USA) as washing agents and eluted in 10mM Tris (pH 8.0).

Whole-genome shotgun metagenomics (WGS) DNA sequencing was performed as previously described [38]. 1 ng of genomic DNA was processed using the Illumina Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Fragmented and tagged DNA was amplified using a limited-cycle PCR program. In this step, index 1(i7) and index 2(i5) were added between the downstream bPCR adaptor and the core sequencing library adaptor, as well primer sequences required for cluster formation. The DNA library was purified using Agencourt® AMPure® XP Reagent (Beckman Coulter, Brea, CA). The DNA library pool was loaded on the Illumina platform reagent cartridge and on the Illumina HiSeq instrument (Illumina, San Diego, CA, USA). A blank composed of only DNA isolation reagents was included in the DNA extraction process and again in the library preparation. ZymoBIOMICS Microbial Community DNA Standard (Zymo Research Corporation, Irvine, CA, USA, Cat#D6305) and a library blank composed of library preparation reagents alone were used as controls. Sequencing reads were demultiplexed

using Illumina Bcl2Fastq 2.18.0.12. Quality of the demultiplexed sequences was verified with FastQC. KneadData was used to remove the human genome contamination from the shotgun metagenome sequencing reads. The taxonomic composition of the filtered reads was characterized with MetaPhlAn2 [39], while the functional genes were annotated with HUMAnN2 [40] following the developers' instructions. The Shannon diversity of samples were calculated with function 'diversity' in R package 'vegan'.

Microbial Signature of Medium Chain Fatty Acids (MCFAs)

HUMANn2 [40] was used to annotate the functional gene families in our shotgun metagenome sequencing dataset using the UniRef90 database. We identified the abundance of acyl-ACP thioesterase genes using the HUMAnN2 gene family annotations. The acyl-ACP thioesterase encoding microbiota that are potentially responsible for MCFAs production were identified based on the taxonomic stratification of acyl-ACP thioesterase at the species level in taxonomy stratified HUMAnN2 gene family annotations. We then selected all the identified acyl-ACP thioesterase encoding bacteria from the taxonomic count table generated with MetaPhlAn2 on the PPCCT shotgun metagenomics sequencing data.

Statistical Analyses

Continuous variables were summarized as mean ± standard deviations (SD) and between-group comparisons were made using Wilcoxon test; categorical variables were summarized with count and proportion and between-group comparisons were made using chi-square test. A logarithmic transformation was used to improve the normality of circulating MCFAs, sugars, ketone bodies and TCA cycle metabolites, MCFAs intake and sugars intake, bacterial abundance, and acyl-ACP thioesterase gene abundance distributions. Geometric means (coefficient of variation) of baseline MCFAs levels and % changes from baseline were calculated and compared between treatment and placebo arms. GLM models adjusting for age, sex, race and baseline levels were used to assess between-group differences on log-transformed data. To rule out the possibility that increased plasma MCFAs came from dietary and supplement intake, we examined whether the increase in plasma MCFAs were caused by the pre- to post-treatment changes in total intake of MCFAs (C7:0 not measurable due to negligible amounts). Pre-treatment intakes of C6:0, C8:0, sucrose, fructose, glucose, maltose, total fructose load, and total glucose load were averaged from two 24-hour dietary recalls at baseline, and post-treatment intakes were averaged from the last two 24-hour dietary recalls during weeks 7 to 12. In addition, we evaluated whether Mg treatment changed the plasma levels of sucrose, fructose, glucose, maltose, total fructose load, and total glucose load compared to the placebo arm. Mediation analyses [41] were conducted with plasma levels of combined C7:0 and C8:0 as a mediator. Pearson correlation was conducted to examine the correlations between changes in Shannon diversity index, abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene, acyl-ACP thioesterase gene abundance/copy number, and the alterations in plasma levels of MCFAs. Based on a positive correlation coefficient ($r \geq 0.2$) following Mg treatment, a microbial signature of MCFAs, i.e. Mg-modifiable signature of MCFAs-related microbiota with acyl-ACP thioesterase gene was developed. All *P* values are two sided and statistical significance was determined using an alpha level of 0.05. The data analyses used software

SAS Enterprise Guide 7.1. To account for multiple comparisons, we set false discovery rate (FDR) at 0.05 using the FDR-controlling procedures by Benjamini and Hochberg [42].

Results

Presented in Table 1 are the comparisons of baseline characteristics between the treatment and placebo arms for 68 participants included in the current study (Supplemental Figure S1). The treatment arm was not significantly different from the placebo arm for baseline demographic variables, including age, sex, race, educational achievement, smoking status, alcohol drinking status, physical activity status, daily intake of total energy, fat, total MCFAs, total Ca, total Mg, intake ratio of Ca to Mg and factors related to cardiometabolic events, including body mass index (BMI), blood pressure and eGFR (estimated glomerular filtration rate).

We found Mg treatment did not significantly affect C6:0 compared to the placebo arm. Mg treatment significantly increased plasma levels of C7:0 and C8:0 by 18.45% and 25.28%, respectively, compared to 14.15% and 10.12% decreases in the placebo arm (P=0.0093, P=0.0046, respectively) (Table 2). Thus, we examined the effect of Mg treatment on combined C7:0 and C8:0 and found Mg treatment significantly increased levels of combined C7:0 and C8:0 by 24.20% compared to a 12.62% reduction in the placebo arm (P=0.0005). The effects remained significant after adjusting for age, sex, race and baseline levels for C7:0, C8:0 and combined C7:0 and C8:0 (P=0.0126, P=0.0162, and P=0.0031, respectively) and additionally controlling FDR at 0.05 (P=0.0324 for both C7:0 and C8:0). In exploratory analysis, we found Mg treatment did not alter the levels of caprate (C10:0) and laurate (C12:0), other two MCFAs (data not shown). In additional analysis, the changes in intakes of C8:0 from both dietary and supplemental sources from before treatment to the end of the trial did not significantly differ by treatment status. We found Mg treatment significantly reduced the plasma levels of free sucrose, total fructose load, and total glucose load compared to the placebo arm (P=0.0216, 0.0444, 0.0444, respectively, for multivariable-adjusted model after additional FDR correction). On the other hand, we did not find the changes in intakes of sucrose, fructose, glucose and maltose from baseline to the end of trial differed between the treatment and placebo arms.

Since we found Mg treatment significantly increased plasma levels of MCFAs (i.e. C7:0, C8:0, and combined C7:0 and C8:0) which improve cellular energy production including ketone bodies [43], we next examined whether the increased MCFAs act as a mediator of Mg treatment on levels of total ketone bodies and TCA cycle metabolites. Table 3 shows results from the mediation analysis. We found that combined C7:0 and C8:0 significantly mediated the effects of Mg treatment on total and individual ketone bodies (P for indirect effect=0.0045, 0.0043 and 0.03, respectively). The indirect effect mediated by combined C7:0 and C8:0 on total ketone bodies accounted for 76.78% of the total effect. However, we did not find combined C7:0 and C8:0 significantly mediate the effect of Mg treatment on fumarate, malate and total TCA cycle metabolites (P for indirect effect =0.17, 0.23, 0.14, respectively). Based on these findings, Figure 1 schematically illustrates our proposed molecular mechanism for the effect of Mg treatment on MCFAs and ketone bodies (see our detailed interpretations in the Discussion section).

Table 4 shows the correlations between changes in gut microbial diversity measured by Shannon diversity index at genus level from stool samples, abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene, acyl-ACP thioesterase gene abundance/copy number in stool and plasma levels of MCFAs. In the treatment arm, the changes in plasma levels of combined C7:0 and C8:0 were significantly positively correlated with alterations in stool Shannon index at genus level (r=0.50, P=0.0026) whereas there were no significant correlations in the placebo arm in stool samples. There were no significant correlations in swab and tissue samples regardless of treatment arm (Data not shown). Based on these findings, we next identified what microbiota in stool samples contribute to the increased levels of C7:0, C8:0 and combined C7:0 and C8:0. Using the shotgun metagenomics sequencing data for stool samples in the PPCCT, we first identified three groups of MCFAs-related microbiota with acyl-ACP thioesterase gene (Supplemental Table S1) for C7:0, C8:0, or combined C7:0 and C8:0. The variations in abundance of bacteria in these three groups were positively correlated (correlation coefficient (r) ≥0.2) with the changes in plasma levels of C7:0, C8:0 and combined C7:0 and C8:0, respectively, in the treatment arm, but not in the placebo arm following Mg treatment. Subsequent, we formed three microbial signatures of MCFAs for C7:0, C8:0 and combined C7:0 and C8:0 by summing up the individual abundance in each group. The changes in the specific microbial signatures in the treatment arm were correlated with circulating C7:0 (r=0.46, p=0.0067), C8:0 (r=0.49, p=0.003) and combined C7:0 and C8:0 (r=0.56, p=0.0006), respectively. The variations in acyl-ACP thioesterase gene abundance/copy number in stool in these three groups were positively correlated with the changes in plasma levels of C7:0(r=0.30, p=0.08), C8:0 (r=0.23, p=0.20) and combined C7:0 and C8:0 (r=0.32, p=0.06), respectively, in the treatment arm, but not in the placebo arm. The correlations for C7:0 and combined C7:0 and C8:0 in the treatment arm were of borderline significance.

Discussion

In this precision-based randomized trial conducted among participants who consumed high Ca:Mg ratio diets, reducing Ca:Mg ratios to around 2.3 through a 12-week personalized Mg treatment significantly increased plasma levels of C7:0 and C8:0 that were not explained by changes in intake levels of MCFAs. Likewise, the significant reduction in circulating levels of sucrose, total fructose load, and total glucose load that accompanied the increase in plasma levels of MCFAs cannot be explained by changes in intakes of sucrose, fructose, glucose, and maltose. Further, we found combined C7:0 and C8:0 partially mediated the effects of Mg treatment on total and individual ketone bodies. Finally, we found the increase in plasma levels of combined C7:0 and C8:0 were positively correlated with increased microbial α-diversity and abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene in stool samples following Mg treatment. Taken together, these novel findings indicate that improvement in Mg availability in the gut that occurs by optimizing Ca:Mg intake ratio leads to increased MCFAs entering the circulation, and in turn, elevated levels of ketone bodies, which is attributable to increased abundance in Mg-modifiable signatures of MCFAs-related microbiota and enhanced gut microbial fermentation and sucrose consumption.

Despite accumulating observations from *in vitro* studies [13,14,16,17,20,44–47], no *in vivo* study has been conducted to examine whether MCFAs can be microbially produced in animals or humans. Our finding is consistent with our hypothesis that optimized dietary Ca:Mg intake ratios could lead to increased circulating level of MCFAs which can be attributed to enhanced production from gut microbial fermentation and sucrose consumption. Previous animal studies found that a Mg-deficient diet leads to lower gut *Bifidobacteria* levels which are associated with increased intestinal permeability and inflammatory response [48] and Mg supplementation enhanced the diversity of gastrointestinal microbiota in animal models [49]. In the current study, we found the changes in MCFAs caused by Mg treatment were significantly and positively correlated with changes in both microbial α-diversity and abundance in Mg-modifiable signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene in stool samples, supporting the involvement of the gut microbiota in the production of MCFAs. Meanwhile, we found there was a significant concomitant reduction in plasma levels of sucrose following the increases in plasma MCFAs induced by Mg treatment. This finding is biologically plausible because microbial fermentation has been suggested to rely on the irreversible conversion of sugar substrates including sucrose into distinct short chain fatty acid (SCFA) and MCFA products [16,17,44–47]. For example, one major member of signatures of MCFAs-related microbiota, *Prevotella buccalis*, characteristically can metabolize sucrose [50]. Sucrose, or table sugar, one common component in Western diet, has been consumed at an increasing trend over the past 100 years [51]. While sucrose is one type of dimeric sugar consisting of one glucose and one fructose, sucrose and free fructose are metabolically equivalent and have similar pathological effects [52]. The health hazards associated with excess intake of sugar (i.e., mainly sucrose and fructose in sugar-sweetened beverages) have been increasingly recognized, including NAFLD [53], metabolic diseases [54], cardiovascular disease [55], colorectal cancer [56], cognitive decline and Alzheimer's disease [57], all of which are also associated with Mg deficiency and may benefit from treatment of MCFAs. Recent evidence shows that sucrose is capable of impeding gut colonization by beneficial microbes through specifically silencing a critical colonization factor called Roc protein [58]. Our findings of significant reduction in plasma levels of free sucrose, total fructose load and total glucose load following Mg treatment indicate that optimizing Ca:Mg ratio in diets may convert excessive detrimental sugars into microbially produced beneficial MCFAs and, in turn, provide potential benefits for ameliorating metabolic diseases linked to Mg deficiency and high sugar consumption. However, future studies are required to confirm this possibility.

An anaerobic environment is a requisite under which MCFAs are favorably produced relative to LCFAs. *Saccharomyces cerevisiae*, a wine fermentative yeast which naturally consumes sucrose as a substrate, produces fatty acid products with a high MCFA/LCFA ratio under anaerobic conditions, but a low ratio (<1) if an aeration strategy is used [59]. Previous studies found the colon has the steepest oxygen gradient in the body, with the oxygen decline from the intestinal mucosa towards the lumen [38,60]. Thus, anaerobic microorganisms which play a key role in fermentation and metabolism of luminal contents (e.g. nutrients or carcinogens) [61–63] are more abundant in luminal than mucosal environments [64]. This may explain why increased levels of MCFAs following Mg treatment are positively correlated with the rise in microbial α-diversity only in stool

samples, but not rectal swab and rectal biopsies. Overall, the correlations between changes in acyl-ACP thioesterase gene abundance in stool and the alterations in plasma MCFA levels in the treatment group are consistent with those between abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene and plasma MCFA levels (Combined C7:0 & C8:0, C7:0, C8:0), respectively. However, the correlations were stronger for the abundance for the signatures of MCFAs-related microbiota than for acyl-ACP thioesterase gene abundance in the treatment arm. One possible cause for the difference is that acyl-ACP thioesterase gene is one necessary factor, but not a sufficient factor to generate C7:0 and/or C8:0. Unlike the abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene, which only include microbiota positively correlating circulating levels of C7:0 and/or C8:0, the gene abundance includes microbiota generating C7:0 and/or C8:0 and those not generating C7:0 and/or C8:0. As a result, the correlations for acyl-ACP thioesterase gene abundance are diluted.

Therapy using MCFAs has consistently been shown to improve cellular energy utilization both in animal models and humans [3–6,8–11], however, the mechanisms are not entirely clear. In the current study, we examined whether the increased MCFAs act as a mediator on levels of total and individual ketone bodies and TCA cycle metabolites that are involved in energy production. Results from our mediation analysis showed that Mg treatment led to increased levels of C7:0 and C8:0, and in turn, an increase in levels of individual and total ketone bodies. This finding further supports the biological mechanism from previous observations suggesting that a medium-chain triglyceride (MCT) ketogenic diet was beneficial for a wide range of neurological and metabolic disorders characterized as dysregulated cellular energy metabolism [3–11]. Our findings that C8:0, considering that C7:0 is in negligible amounts in natural sources, rather than any other MCFAs (i.e., C6:0, C10:0 and C12:0), mediated the Mg-induced effects on ketone bodies. C8:0 is the most potent ketogenic MCFA, which generating three times more ketones than C10:0 and about six times more than C12:0 [65]. Our findings also provide a novel mechanism through which optimal Mg status reduce risks of a variety of metabolic and neurodegenerative disorders [66–72].

One promising finding from the current study is that in addition to C8:0, we found Mg treatment also significantly increased plasma levels of C7:0, a MCFA with an odd number of carbons. C7:0 possesses unique beneficial features that even-numbered MCFAs (e.g. C8:0) do not [73–75]. Previous studies found C7:0 has anti-inflammatory effects and can be used for treating psoriasis, allergies and autoimmune diseases [75]. Regardless of their nutritional and pharmaceutical applications, the amounts of nature-originated odd-numbered MCFAs in dairy products and tropical oils are negligible [76]. Therefore, it is unlikely that the increase in plasma levels of C7:0 is caused by dietary intake or supplemental use of C7:0. In addition, dietary intakes of both C8:0 and sucrose did not change significantly from baseline to the end of the trial. Thus, our findings indicate that Mg treatment not only increases even-numbered MCFAs, but also increases the microbial production of odd-numbered MCFAs (i.e. C7:0). Collectively, our findings indicate the synergistic use of Mg and MCFAs, especially C8:0, as a potential therapy for the prevention and treatment of a number of neurodegenerative and metabolic diseases.

This study has several strengths, including the double-blinded randomized trial design. Furthermore, a precision-based design was used. Intakes of Mg and Ca from both diet and supplements were measured twice before and four times during the treatment and a personalized dosing strategy of Mg supplementation was administered to each participant. In addition, we had a high compliance with the pill regimen for both the treatment and placebo arms based on pill counts (96.70% ± 7.61% and 96.60% ± 6.87%, respectively; $P_{\text{difference}}$= 0.91) and the dropout rate was very low (4%).

There are some limitations for our study. Although we identified Mg-modifiable signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene, and observed its correlation with changes in plasma MCFAs, the microbiome associated MCFAs production in the human gut and underlying mechanisms are not well understood. Other microorganisms may also contribute to the MCFAs production process. Furthermore, we cannot rule out the possibility of impact of other metabolic pathways involved in MCFAs production and release. In particular, Mg may increase the enzyme activity of acyl-ACP thioesterase, but not the abundance of acyl-ACP thioesterase encoding bacteria. In these cases, the metagenomic approach used in the current study cannot detect these changes, neither can a transcriptomics approach. Future experiments using RT PCR or RNAseq are needed to further verify the findings that acyl-ACP thioesterase is expressed by members of the stool microbiota in the Mg supplemented arm compared to the others. Secondly, the plasma levels of MCFAs are influenced by homeostasis between synthesis and degradation, which may not accurately reflect the amount of MCFAs that were initially produced by microbiota in the human gut. Furthermore, the intestinal absorption of nutrients including sucrose is regulated by gut mucosal barrier function and intestinal permeability so that it is not clear whether the reduction in plasma levels of sucrose after Mg treatment is due to increased consumption of sucrose by gut microbiota or reduced absorption following decreased intestinal permeability. Lastly, the sample size is relatively small in our study. However, the effects of Mg on MCFAs are still highly significant even after the multiple comparison corrections indicating the effects of Mg treatment on MCFAs are robust. Furthermore, considering the results from pathway analyses including mediation analysis are highly consistent, the findings from our study are unlikely to be solely due to chance. In addition, it is possible that the non-significant effects of Mg treatment on TCA cycle metabolites may be due to a small sample size. Future studies are necessary to further address these important scientific inquiries.

In summary, optimizing Ca:Mg intake ratios to around 2.3 through 12-week personalized Mg supplementation leads to increased circulating levels of MCFAs (i.e. C7:0 and C8:0), which are attributable to enhanced production from gut microbial fermentation and, maybe, sucrose consumption. These findings, if confirmed, have significant implications for the development of novel and effective interventions for a large variety of human diseases, such as neurodegenerative, metabolic, and immunomodulatory disorders, and improved animal health, particularly survival in baby animals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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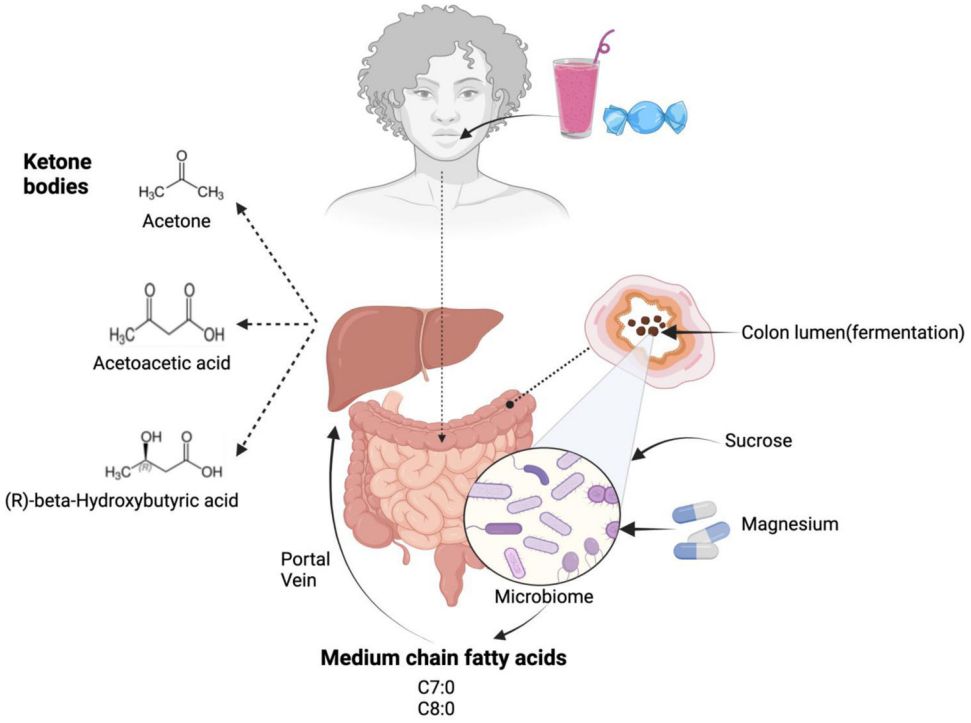

Figure 1.
Proposed Molecular Mechanism

Table 1

Characteristics of treatment vs. placebo arms at baseline, PPCCT

	Placebo *n*=34	Treatment *n*=34	*P* value
Age, year	63.24±7.65	61.35±9.00	0.32
Sex - male (%)	55.88	47.06	0.47
Race - white (%)	97.06	100	0.31
Education under college (%)	5.88	11.76	0.45
Smoking status (%)			0.61
Never	55.88	44.12	
Ever	38.24	47.06	
Current	5.88	8.82	
Drinking status (%)			0.15
Never	47.06	26.47	
Ever	14.71	29.41	
Current	38.24	44.12	
Physically active in ≥ 2 days per week (%)	82.35	64.71	0.10
Body mass index (BMI), kg/m^2	29.54±4.77	29.61±6.13	0.98
Systolic blood pressure, mmHg	127±14	127±14	0.95
Diastolic blood pressure, mmHg	73.39±7.68	71.97±7.79	0.37
eGFR, mL/(min·1.73m^2)	77.41±14.74	82.71±15.32	0.10
Daily nutrients intake			
Total energy intake, kcal/day	2060±535	2000±478	0.32
Fat, g/day	86.82±31.21	79.06±28.70	0.13
Total MCFA intake (C6:0-C12:0), g/day	2.67±1.92	2.35±1.72	0.40
Caproate (C6:0), g/day	0.45±0.30	0.34±0.25	0.07
Caprylate (C8:0), g/day	0.36±0.24	0.31±0.25	0.28
Caprate (C10:0), g/day	0.68±0.42	0.57±0.40	0.15
Laurate (C12:0), g/day	1.18±1.23	1.13±0.93	0.96
Total Ca intake, mg/day	1225±462	1223±404	0.84
Total Mg intake, mg/day	354±173	355±88	0.22
Ca:Mg intake ratio	3.64±0.97	3.46±0.91	0.39

Continuous variables were presented as mean±SD and *P* values were calculated using Wilcoxon test; categorical variables were presented as % and *P* values were calculated using Pearson chi-square test.

eGFR, estimated glomerular filtration rate; MCFA, medium chain fatty acid; PPCCT, Personalized Prevention of Colorectal Cancer Trial.

Table 2

Mg treatment and changes in circulating levels and intakes of MCFAs and sugars, PPCCT

	Placebo arm[a]		Treatment arm[a]		*P* values		
	Baseline level *n*=34	% Change	Baseline level *n*=34	% Change	P_1	P_2	P_3
Plasma MCFAs							
Caproate (C6:0)	0.81 (0.82)	30.14%	0.65 (0.66)	14.69%	0.54	0.16	0.16
Heptanoate (C7:0)	0.75 (0.54)	−14.15%	0.70 (0.44)	18.45%	0.0093**	0.0126*	0.0324*
Caprylate (C8:0)	1.19 (0.63)	−10.12%	0.96 (0.35)	25.28%	0.0046**	0.0162*	0.0324*
C7:0 + C8:0	2.06 (0.51)	−12.62%	1.70 (0.32)	24.20%	0.0005***	0.0031**	-
Plasma sugars							
Free sucrose	0.62 (2.17)	36.46%	0.72 (2.52)	−55.04%	0.002**	0.0036**	0.0216*
Free fructose	0.95 (0.33)	2.11%	1.10 (0.30)	−6.36%	0.27	0.68	0.68
Free glucose	1.04 (0.15)	0.56%	1.00 (0.10)	−0.58%	0.69	0.43	0.64
Free maltose	0.99 (0.98)	−17.41%	1.18 (1.13)	−15.60%	0.91	0.57	0.68
Total fructose load[b]	1.94 (0.68)	29.54%	2.23 (0.72)	−28.38%	0.0063**	0.0222*	0.0444*
Total glucose load[c]	2.01 (0.65)	17.08%	2.13 (0.71)	−25.24%	0.0080**	0.0150*	0.0444*
Daily intake[d]							
Caprylate (C8:0)	0.29 (0.86)	−35.59%	0.25 (0.75)	−19.14%	0.31	0.42	0.88
Sucrose	34.83 (0.96)	−20.30%	34.10 (1.17)	−15.16%	0.92	0.78	0.88
Fructose	15.57 (0.75)	−25.88%	19.15 (0.83)	−18.43%	0.75	0.62	0.88
Glucose	18.28 (0.71)	−9.41%	17.80 (0.77)	−9.27%	0.93	0.88	0.88
Maltose	2.64 (0.74)	−29.22%	2.15 (1.06)	−1.14%	0.23	0.21	0.88

[a]Data are presented in geometric mean (coefficient of variation) or percent change in geometric mean.

[b]Total fructose load was defined as the sum of free fructose and free sucrose because sucrose consists of one fructose and one glucose.

[c]Total glucose load was defined as the sum of free glucose and free sucrose.

[d]Daily intake incorporated dietary intake from food and supplement use.

P_1: *P* value for unadjusted GLM model using log-transformed data; P_2: *P* value for GLM model adjusting for age, sex, race and baseline level using log-transformed data; P_3: *P* value for GLM model adjusting for age, sex, race and baseline level after FDR correction using log-transformed data. Mg, magnesium; MCFA, medium chain fatty acid; PPCCT, Personalized Prevention of Colorectal Cancer Trial.

* $P<0.05$,

** $P<0.01$,

*** $P<0.001$.

Table 3

Total, direct and indirect effects of Mg treatment on ketone bodies and TCA cycle metabolites, PPCCT

	Total effect		Direct effect		Indirect effect	
Outcomes (mediator: combined C7:0 and C8:0)[d]	**Point estimate (95% CI)[c]**	***P* value**	**Point estimate (95% CI)[c]**	***P* value**	**Point estimate (95% CI)[c]**	***P* value**
Total ketone bodies [a]	0.53 (0.11, 1.00)	0.02	0.12 (−0.28, 0.49)	0.58	0.41 (0.14, 0.75)	0.0045 **
Acetoacetate	0.38 (−0.08, 0.82)	0.10	−0.03 (−0.43, 0.35)	0.88	0.42 (0.17, 0.77)	0.0043 **
3-hydroxybutyrate (BHBA)	0.51 (0.09, 1.00)	0.03	0.25 (−0.16, 0.59)	0.31	0.26 (0.02, 0.66)	0.03 *
Total TCA cycle metabolites [b]	0.37 (−0.07, 0.83)	0.12	0.21 (−0.30, 0.77)	0.41	0.16 (−0.07, 0.44)	0.17
Fumarate	0.42 (−0.03, 0.91)	0.08	0.28 (−0.22, 0.84)	0.26	0.13 (−0.07, 0.41)	0.23
Malate	0.26 (−0.23, 0.74)	0.28	0.09 (−0.48, 0.68)	0.73	0.17 (−0.04, 0.44)	0.14

[a] Total ketone bodies=acetoacetate+3-hydroxybutyrate. All values are log transformed.

[b] Total TCA cycle metabolites=fumarate+malate. All values are log transformed.

[c] 95%CIs are bootstrap bias corrected.

[d] Mediation analyses were conducted with plasma levels of combined C7:0 and C8:0 as a mediator.

* *P*<0.05,

** *P*<0.01,

*** *P*<0.001.

Mg: magnesium. TCA cycle, tricarboxylic acid cycle; PPCCT, Personalized Prevention of Colorectal Cancer Trial.

Table 4

Changes in gut microbial α-diversity index, abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene, and acyl-ACP thioesterase gene abundance with plasma MCFAs by Mg treatment

Plasma MCFAs	Mg treatment			Placebo		
	N	r	*P*	N	r	*P*
Shannon diversity index in stool						
Combined C7:0 and C8:0	34	0.50	0.0026**	30	0.003	0.99
C7:0		0.51	0.0023**		0.003	0.99
C8:0		0.34	0.0497*		0.03	0.87
Abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene						
Combined C7:0 and C8:0	34	0.56	0.0006***	30	−0.05	0.79
C7:0		0.46	0.0067**		0.04	0.81
C8:0		0.49	0.003**		−0.08	0.69
Acyl-ACP thioesterase gene abundance/copy number						
Combined C7:0 and C8:0	34	0.32	0.06	30	−0.21	0.26
C7:0		0.30	0.08		−0.22	0.24
C8:0		0.23	0.20		−0.19	0.31

*$P<0.05$,

**$P<0.01$,

***$P<0.001$. Abundance for the signatures of MCFAs-related microbiota with acyl-ACP thioesterase gene, and acyl-ACP thioesterase gene abundance in stool were log-transformed. Microbial signature of MCFAs was the sum of the relative abundance of all related bacteria. Pearson correlation was used for detecting a correlation.

Mg, magnesium; MCFA, medium chain fatty acid; PPCCT, Personalized Prevention of Colorectal Cancer Trial.

Microbial signature of C7 related bacteria included *Prevotella buccalis, Clostridium hylemonae, Ruminococcus obeum, Dorea formicigenerans, Catenibacterium mitsuokai*, and *Pyramidobacter piscolens*.

Microbial signature of C8 related bacteria included *Prevotella buccalis, Prevotella copri, Eubacterium ventriosum*, and *Pyramidobacter piscolens*.

Microbial signature of combined C7 and C8 related bacteria included *Prevotella buccalis, Prevotella copri, Ruminococcus obeum, Coprococcus catus*, and *Pyramidobacter piscolens*.